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Analytical Methods

Comparative HPLC/ESI-MS and HPLC/DAD study of different populations of cultivated, wild and commercial *Gentiana lutea* L.

Ahmed M. Mustafa [a,1], Giovanni Caprioli [a,1], Massimo Ricciutelli [a], Filippo Maggi [a], Rosa Marín [b], Sauro Vittori [a], Gianni Sagratini [a,*]

[a] School of Pharmacy, University of Camerino, Via Sant'Agostino 1, 62032 Camerino, Italy
[b] Department of Analytical Chemistry, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

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ABSTRACT

The root of *Gentiana lutea* L., famous for its bitter properties, is often used in alcoholic bitter beverages, food products and traditional medicine to stimulate the appetite and improve digestion. This study presents a new, fast, and accurate HPLC method using HPLC/ESI-MS and HPLC/DAD for simultaneous analysis of iridoids (loganic acid), secoiridoids (gentiopicroside, sweroside, swertiamarin, amarogentin) and xanthones (isogentisin) in different populations of *G. lutea* L., cultivated in the Monti Sibillini National Park, obtained wild there, or purchased commercially. Comparison of HPLC/ESI-MS and HPLC/DAD indicated that HPLC/ESI-MS is more sensitive, reliable and selective. Analysis of twenty samples showed that gentiopicroside is the most dominant compound (1.85–3.97%), followed by loganic acid (0.11–1.30%), isogentisin (0.03–0.48%), sweroside (0.05–0.35%), swertiamarin (0.08–0.30%), and amarogentin (0.01–0.07%). The results confirmed the high quality of the *G. lutea* cultivated in the Monti Sibillini National Park.

1. Introduction

Gentian (*Gentiana lutea* L.) is a widely distributed herbaceous species of the *Gentianaceae* family. It is native to the mountains of central and southern Europe, preferring calcareous soils, and is found naturally (wild) in France, Spain and the Balkan mountains. The plant is under wildlife protection in Europe, and is cultivated particularly in Germany and France. It is dried directly after the harvesting to avoid fermentation, which drastically reduces the extract content and leads to changes in colour (Blaschek et al., 2006; European Medicines Agency (EMA), 2009; Hänsel & Sticher, 2007).

The root of *G. lutea* is famous for its bitter properties and is often used in alcoholic bitter beverages, food products and traditional medicine to stimulate the appetite and improve digestion (Carnat et al., 2005; European Medicines Agency, 2009; Mustafa et al., 2014). This is mainly due to its content of bitter-tasting secoiridoids, in particular swertiamarin, gentiopicroside, sweroside and amarogentin. The quality of gentian root is evaluated by assessing its content of gentiopicroside, a major bitter principle, which is strongly affected by the plant's developmental stage and its environment, as well as the preparation process (Ando et al., 2007; Carnat et al., 2005).

Variations of levels of each compound of *G. lutea* are related to geographical origin, stage of development and age of roots. Some authors have reported an increase in gentiopicroside levels during plant growth, followed by a decrease after flowering until dehiscence of fruits (Franz, Franz, Fritz, & Schultze, 1985; Rossetti, Lombard, Sancin, Buffa, & Borgarello, 1981). Other authors have analysed gentiopicroside levels according to altitude, but reported conflicting results (Rossetti, Lombard, Sancin, Buffa, & Menghini, 1984; Schultze & Franz, 1979). Amarogentin levels were found to vary with the ecotype, from trace amounts to low percentages (Franz & Fritz, 1975; Sancin, Rossetti, Lombard, Buffa, & Lanzone, 1985). It has been observed that the levels of amarogentin and gentiopicroside changed inversely with stage of development and sugar accumulation in roots (Franz et al., 1985). Results of a study carried out on cultivated plants indicated high amounts of gentiopicroside and amarogentin in the one-year roots, and a decrease in roots cultivated for 5 years (Hayashi, Minamiyama, Miura, Yamagishi, & Kaneshina, 1990). In contrast, xanthone levels did not change significantly with either altitude or age of roots; a maximum xanthone level was observed during the flowering period and a minimum one during the non-vegetative period (Rossetti et al., 1984; Verney, Ozenda, & Debelmas, 1972).

* Corresponding author. Tel.: +39 0737402238; fax: +39 0737637345.
E-mail address: gianni.sagratini@unicam.it (G. Sagratini).
[1] These authors equally contributed to the article.

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Numerous pharmacological effects have been attributed to the bitter tasting secoiridoids of *G. lutea*, among them stomachic, digestive, cholagogic, hepatoprotective, and wound-healing activities (European Medicines Agency, 2009; Newall, Anderson, & Phillipson, 1996; Ozturk, Korkmaz, Ozturk, & Baser, 2006). Secoiridoids, iridoids and xanthones are the main active constituents of the Gentian plant, but the secoiridoids (gentiopicroside, amarogentin, swertiamarin and sweroside) are the most important because they are responsible for the bitter taste of the plant and bring about a reflex excitation of the taste receptors that increases secretion of saliva and digestive juices and consequently stimulates the appetite and improves digestion (European Medicines Agency, 2009; Kusar, Sircelj, & Baricevic, 2010). Single constituents have been identified as active principles. For example, loganic acid showed anti-inflammatory activity (Recio, Giner, Manez, & Rios, 1994) and isogentisin revealed potent MAO inhibition (Haraguchi et al., 2004; Suzuku et al., 1978). Also, *G. lutea* extracts showed antioxidant and anti-inflammatory activities (Mathew, Taranalli, & Torgal, 2004; Nastasijevic et al., 2012).

Analysis of some components of *G. lutea* roots by HPLC has been described in a number of publications (Arino, Arberas, Leiton, Renobales, & Dominguez, 1997; Carnat et al., 2005; Chen, Zhang, Guan, & Yu, 2004; Chueh, Chen, Sagare, & Tsay, 2001; Jiang et al., 2005; Szucs, Danos, & Nyiredy, 2002; Yang et al., 2009). Only two reports described the simultaneous quantitative determination of secoiridoids, iridoids and xanthones, and they only examined commercial gentian root samples by HPLC/DAD, using HPLC/MS only for identification, and not for quantitation (Aberham, Pieri, Croom, Ellmerer, & Stuppner, 2011; Aberham, Schwaiger, Stuppner, & Ganzera, 2007).

The aim of the present work was to develop a new fully validated HPLC/ESI-MS method for the simultaneous determination of iridoids, secoiridoids and xanthones in *G. lutea* samples that were cultivated for the first time in the Monti Sibillini National Park (Italy), collected wild in the March-Umbria area, or bought in herbal shops, and to evaluate the quality of the *G. lutea* L. cultivated in the Park. To our knowledge, this is the first time that HPLC/ESI-MS was used for simultaneous quantification of these compounds in *G. lutea*.

2. Experimental

2.1. Plant Materials

The roots of *G. lutea* L. that were cultivated for 4 years in the Monti Sibillini National Park (Italy) were collected in Pian Perduto (1334 m, Castel Sant'Angelo sul Nera, Macerata, 1334 m a.s.l., samples GL-1 and GL-2) at the end of October 2013. Wild roots of *G. lutea* subsp. *symphyandra* were collected in the same period in five different localities of the Park at different altitudes ranging from 1277 m to 1520 m as follows: Pian Grande (1277 m a.s.l., N 42°47′23″ E 13°11′03″, Norcia, Perugia, samples GL-3 and GL-4), Santa Maria Maddalena (1285 m a.s.l., N 43°02′18″ E 13°13′00″, Acquacanina, Macerata, samples GL-5 and GL-6), Pian Perduto (1334 m a.s.l., N 42°50′25″ E 13°12′06″, Castel Sant'Angelo sul Nera, Macerata, samples GL-7 and GL-8), Prati di Ragnolo (1511 m a.s.l., N 43°00′58″ E 13°13′71″, Bolognola, Macerata, samples GL-9 and GL-10), and Pizzo Tre Vescovi (1520 m a.s.l., N 42°58′07″ E 13°14′10, Bolognola, Macerata, samples GL-11 and GL-12). For each locality, samples were from two different individuals. Plant samples were botanically confirmed by Dr. Filippo Maggi, and the voucher specimens deposited in the *Herbarium Universitatis Camerinensis* of School of Biosciences and Veterinary Medicine (University of Camerino, Italy). Root samples were cleaned and cut into small pieces, then dried using an electric oven at 45 °C for 6 days in a process termed artificial drying. The commercial roots were either kindly provided by the Varnelli S.p.A. company (Marche, Italy, samples GL-13 and GL-14) or purchased from herbal shops in Camerino (Marche, Italy, samples GL-15 and GL-16), Foggia (Puglia, Italy, sample GL-17), Castelraimondo (Marche, Italy, sample GL-18), Matelica (Marche, Italy, sample GL-19) and Ascoli Piceno (Marche, Italy, sample GL-20).

2.2. Chemicals

All the chemicals used were of HPLC or analytical grade. The analytical standards of loganic acid, swertiamarin, sweroside, gentiopicroside, amarogentin and isogentisin were purchased from PhytoLab (Germany). The purity of all standard compounds was ⩾97% (determined by HPLC). HPLC-grade acetonitrile (⩾99.9) and formic acid were purchased from Sigma–Aldrich (Milan, Italy). Deionised water (>18-MΩ cm resistivity) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents and solutions were filtered before HPLC analysis through 0.45-μm PTFE filters purchased from Phenomenex (Bologna, Italy).

2.3. HPLC sample preparation

The artificially dried roots were ground using an electric mill with a 2 mm pore size. The finely powdered root material (10 mg) was extracted with 10 ml methanol by sonication (45 min, at room temperature) by obtaining a drug/extract ratio of 35.2% (w/w). After centrifugation at 5000 rpm for 10 min with a Thermo Scientific IEC CL10 Centrifuge from Thermo Electron Industries SAS (Chateau-Gontier, France), the extracts were transferred to a 10 ml volumetric flask, which was then filled up to the final volume with extraction solvent. The sample solutions were filtered through a 0.45 μm pore size nylon membrane filter (Phenex, Phenomenex, Torrance, CA, USA) before injection into the HPLC. All samples were stored in a refrigerator at the temperature of 4 °C until analysis. Each sample was analysed in triplicate.

2.4. Instruments and conditions

HPLC/DAD and HPLC/ESI-MS (ion trap) studies were performed using an Agilent 1100 (Santa Clara, CA, USA) series instrument, made from an autosampler, a binary solvent pump, with a diode–array detector (DAD) and a mass spectrometer detector (MSD) Trap SL equipped with an electrospray ionisation (ESI) interface operating in negative ionisation (NI) mode. The separation was achieved on a Synergi Polar–RP C18 (4.6 mm × 150 mm, 4 μm) analytical column from Phenomenex (Chesire, UK). The column was preceded by a Polar RP security guard cartridge (4 mm × 3 mm ID). The mobile phase for HPLC/DAD and HPLC/ESI-MS analyses was aqueous-formic acid (99.9–0.1%) (A) and acetonitrile (B) working in the gradient mode at a flow rate of 0.6 ml min^{-1}. The solvent composition varied as follows: 0–7 min, 20% B; 7–15 min, 20–90% B; 15–18 min, 90% B; 18–25 min, 90–20% B. The column temperature was set at 11 °C and the injection volume was 10 μl.

HPLC/DAD analysis was performed monitoring four different wavelengths: 232 for loganic acid, swertiamarin and amarogentin; 246 nm for sweroside; 258 nm for isogentisin and 275 nm for gentiopicroside.

In HPLC/ESI-MS, the column outlet was connected to the electrospray sample inlet. The electrospray (ESI) source operated in negative ionisation (NI) mode. Optimisation of the HPLC/ESI-MS conditions was carried out by flow injection analysis (FIA) of the analytes (10 μl of a 50 μg ml^{-1} individual standard solutions). The optimum ESI interface conditions were as follows: vaporizer

temperature, 325 °C; nebulizer gas (nitrogen) pressure, 50 psi; drying gas (nitrogen) flow rate, 11 ml min^{-1}; temperature, 350 °C; and capillary voltage, 3500 V. Mass scan range was set in the range of *m*/*z* 100–1000. Extract ion chromatograms (EIC) from total ion chromatograms (TIC) were used for the analysis.

2.5. Multivariate analysis

To reveal the relationship among different gentian samples according to compositions of secondary metabolites, and to identify the main constituents influencing variability, the composition data matrix of twenty samples (6 variables × 20 samples = 120 data) was analysed using principal component analysis (PCA) with *STATISTICA 7.1* (Stat Soft Italia srl, 2005, www.statsoft.it). Eigenvalues were calculated using a covariance matrix among 6 chemical compounds as input, and the two-dimensional PCA biplot, including both samples of different origin and compounds, was generated.

3. Results and discussion

3.1. Optimisation of chromatographic conditions

3.1.1. HPLC/DAD

The selection of the chromatographic conditions was guided by the need to obtain chromatograms with the best resolution of adjacent peaks in a short time of analysis. In the current study, different detection wavelengths (λ = 232, 246, 258 and 275 nm) were used to monitor all compounds simultaneously in a single run to provide sufficient sensitivity for each analyte. The compounds in the samples were identified by comparing both retention times and UV spectra with those of the authentic standards. In focusing on the analysis of iridoids (loganic acid), secoiridoids (swertiamarin, sweroside, gentiopicroside, amarogentin) and xanthones (isogentisin) in *G. lutea*, we encountered difficulties caused by the wide polarity range of the analytes of interest by the very close structural resemblance among some of the compounds (Fig. 1). After trying different organic solvents such as methanol and acetonitrile, with aqueous formic acid solution, it was found that the mixture of aqueous formic acid solution (99.9–0.1%) and acetonitrile was an ideal mobile phase for the analysis and separation (see chromatogram Fig. 2A). An aqueous acetic acid solution (99.9–0.1%) and ammonium formate buffer with acetonitrile also showed good results, though not as good as those yielded by the aqueous formic acid solution (99.9–0.1%). We studied the effect of column temperature, examining room temperature, 30, 15 and 11 °C, and found that at room temperature and 30 °C, the peaks of sweroside and gentiopicroside were totally coeluted; at 15 °C, the column demonstrated a partial coelution of the same two peaks, but when the column temperature was kept at 11 °C, all peaks were well separated. The flow rates of 0.5, 0.6 and 0.7 ml/min were tested, with the flow rate of 0.6 ml/min showing the best separation. Moreover, considering the different and wide degrees of polarity of the 6 standards, gradient elution was used to achieve better separation. Under the optimum gradient conditions (0–7 min, 20% B; 7–15 min, 20–90% B; 15–18 min, 90% B; 18–25 min, 90–20% B), the baseline separation of the peaks of these 6 compounds was achieved. Other gradient conditions caused poor separation of some peaks, especially sweroside and gentiopicroside, or extended the run time.

3.1.2. HPLC/ESI-MS

The HPLC/ESI-MS (ion trap mass spectrometer) experiments used the same chromatographic conditions chosen for HPLC/DAD analyses.

The HPLC/ESI-MS analysis was performed in Scan, and for optimum MS results, ionisation was performed in negative ESI mode. In this experiment, the deprotonated molecular ions $[M-H]^-$ of loganic acid, amarogentin and isogentisin at *m*/*z* 375, 585 and 257, respectively and the $[M+HCOOH-H]^-$ ions of swertiamarin, sweroside and gentiopicroside at *m*/*z* 419, 403 and 401, respectively, were chosen as the quantitative analysis ions through EIC from the TIC chromatogram. These distinct adducts or quasi-molecular ions were the base peak for the analysed compounds, and were in good agreement with the reported data (Aberham et al., 2007; Szucs et al., 2002). In HPLC/ESI-MS spectra, the molecular ion of each compound was well matched with chemical structures. Fig. 2 depicts the HPLC/ESI-MS (Scan) chromatograms of the standard mixture of the analysed compounds at a concentration of

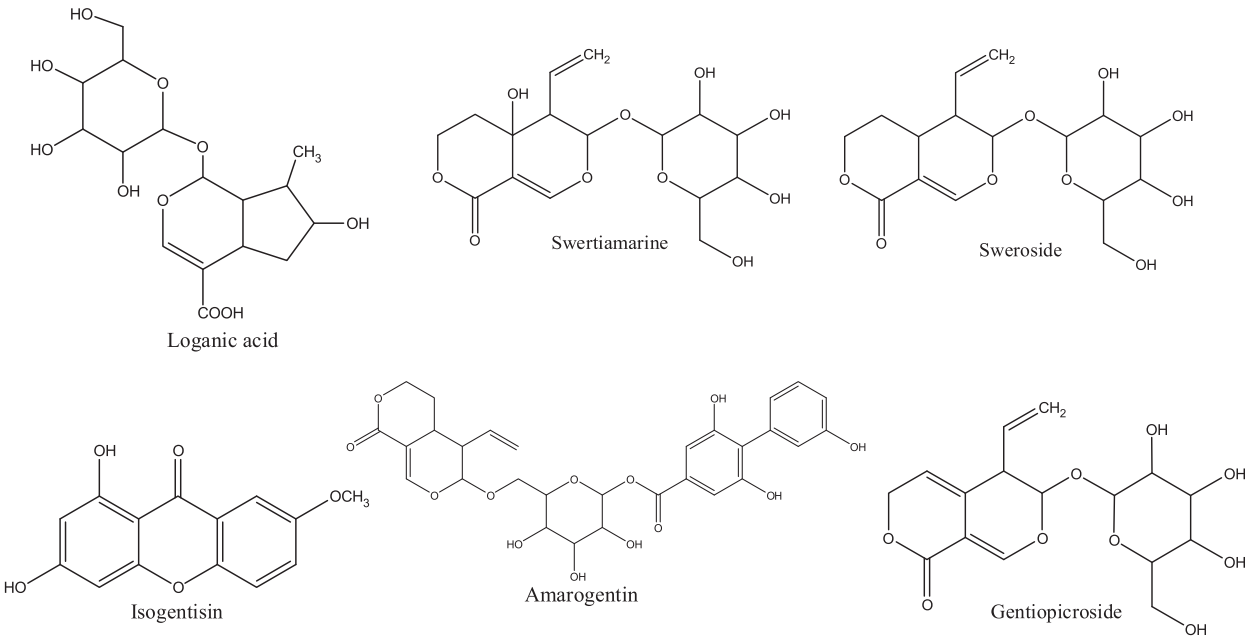

Fig. 1. Chemical structures of the monitored compounds.

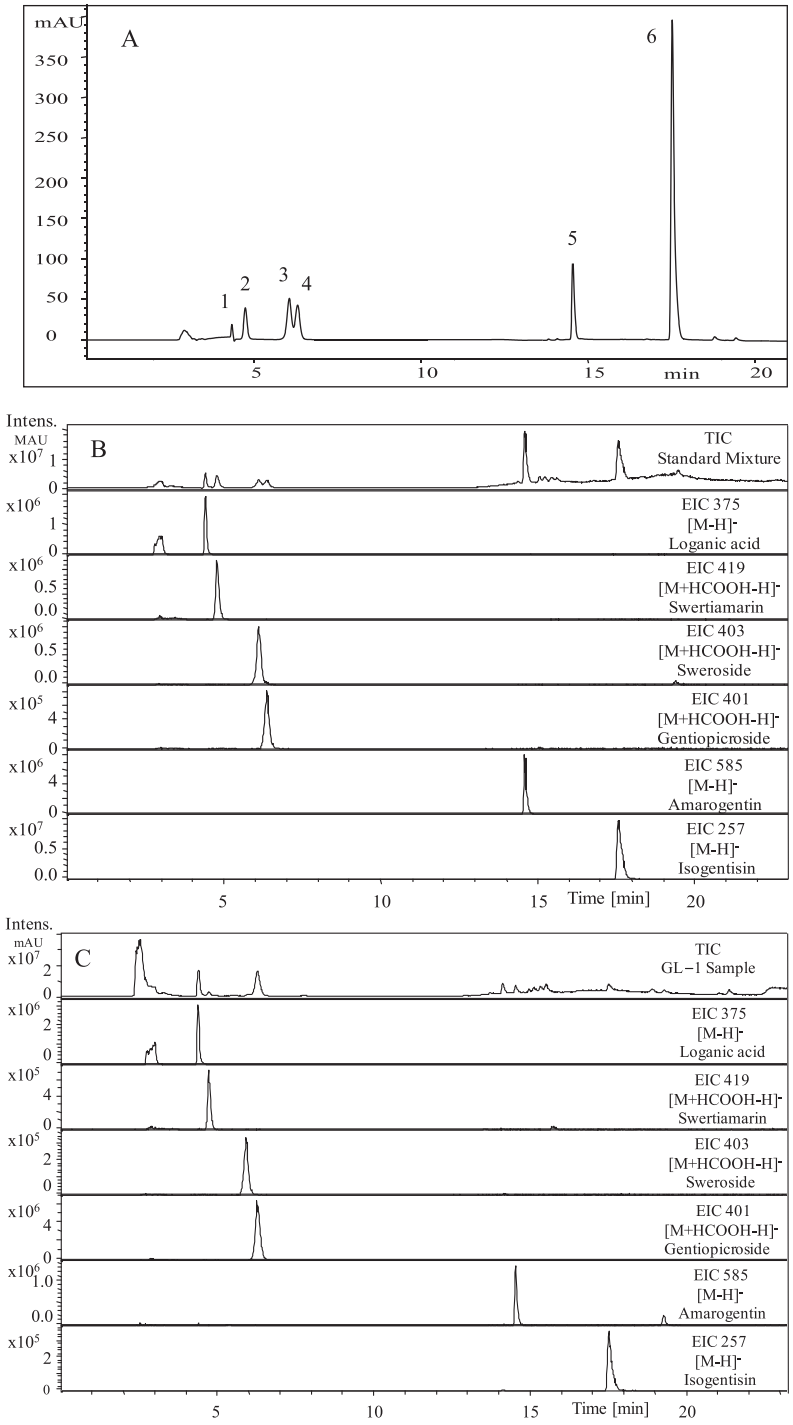

Fig. 2. HPLC/DAD and LC/ESI-MS (Scan) chromatograms: (A) HPLC/DAD chromatogram of standard mixture at 258 nm (25 μg/ml); (B) LC/ESI-MS chromatogram of standard mixture at (5 μg/ml); (C) LC/ESI-MS chromatogram of cultivated *G. lutea* L. sample GL-1.

5 μg/ml (Fig. 2B) and a cultivated gentian sample (Fig. 2C) (both total ion current (TIC) and extracted ion current (EIC) chromatograms) in the negative ESI-MS mode.

3.2. In-house method validation

3.2.1. HPLC/DAD

After optimising the chromatographic conditions, the HPLC/DAD method was validated in terms of precision, accuracy, linearity, limits of detection (LODs) and limits of quantification (LOQs) (Table 1). The intra-day precision of the HPLC/DAD method was validated with the injection of the standard mixture solution under the selected optimal conditions five times a day. For inter-day precision, measurements were conducted once a day on three consecutive days. All of the precision measurements were expressed as relative standard deviations (RSDs) and ranged from 0.23 to 1.43; the precision and the repeatability of the concentration proved to be very good. Accuracy was determined by spiking sample GL-9 (Fargno) with three concentrations of standard compounds (low, medium, high spike). For this purpose, known amounts of the standard mixture solution were added to the dry, powdered plant material, which was then extracted and assayed as described before. The

Table 1
Concentration range, linearity (Correlation coefficient; R^2), Limits of detection (LODs), Limits of quantification (LOQs) of the six analysed compounds by HPLC/DAD and LC/ESI-MS (relative standard deviation RDS% are given in parenthesis).

HPLC/DAD									LC/ESI-MS (ion trap)					
Name	Conc. range (μg/ml) 8 points	R^2	LOD (μg/ml)	LOQ (μg/ml)	Recovery % (n = 3)				R^2	LOD (μg/ml)	LOQ (μg/ml)	Recovery % (n = 3)		
					Level of spiking 0.5 μg/ml	Level of spiking 1 μg/ml	Level of spiking 5 μg/ml	Conc. range (μg/ml) 5 points				Level of spiking 0.5 μg/ml	Level of spiking 1 μg/ml	Level of spiking 5 μg/ml
Loganic acid	0.5–100	0.9997	0.25	0.75	104.67 (1.93)	100.87 (2.56)	100.61 (0.38)	0.1–10	0.9996	0.025	0.075	99.09 (2.72)	94.85 (4.36)	92.61 (4.57)
Swertiamarin	0.5–100	0.9999	0.10	0.30	95.45 (4.76)	95.06 (3.77)	99.30 (0.88)	0.1–10	0.9998	0.025	0.075	101.86 (3.52)	105.16 (4.47)	93.98 (3.69)
Sweroside	0.5–100	0.9998	0.10	0.30	95.76 (4.77)	104.76 (2.92)	101.06 (0.78)	0.1–10	0.9994	0.025	0.075	105.15 (1.80)	96.68 (5.43)	94.51 (3.81)
Gentiopicroside	0.5–100	0.9999	0.10	0.30	105.28 (4.35)	94.69 (4.45)	95.63 (4.29)	0.1–10	0.9998	0.025	0.075	104.93 (5.30)	104.70 (5.62)	107.48 (1.28)
Amarogentin	0.5–100	0.9999	0.02	0.05	101.11 (1.44)	95.68 (1.80)	97.28 (1.07)	0.1–10	0.9996	0.01	0.03	95.70 (3.79)	94.11 (4.79)	92.58 (1.72)
Isogentisin	0.5–100	1	0.02	0.05	97.84 (2.02)	99.37 (0.92)	100.41 (0.26)	0.1–5	0.9992	0.01	0.03	94.89 (4.12)	93.11 (5.04)	92.09 (1.84)

LOD (limit of detection) = 3 × signal-to-noise (S/N) ratio.
LOQ (limit of quantitation) = 10 × signal-to-noise (S/N) ratio.

Table 2
Quantitative determination of the analysed compounds in 20 *Gentiana lutea* L. samples (μg/ml and percent %) by LC/ESI-MS; relative standard deviations were in a range from 0.02 to 5.46 (n = 3).

Compound sample	Loganic acid		Swertiamarin		Sweroside		Gentiopicroside		Amarogentin		Isogentisin	
	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%	μg/ml	%
GL-1	13012.73	1.30	2548.76	0.25	1528.59	0.15	35278.83	3.53	740.84	0.07	1795.98	0.18
GL-2	11548.46	1.15	2444.14	0.24	1484.69	0.15	33052.33	3.31	690.60	0.07	1526.48	0.15
GL-3	1998.46	0.20	1923.72	0.19	1270.49	0.13	23580.20	2.36	202.64	0.02	4298.19	0.43
GL-4	1793.74	0.18	1907.33	0.19	1132.42	0.11	23023.45	2.30	207.12	0.02	4323.97	0.43
GL-5	2050.94	0.21	2217.90	0.22	790.35	0.08	35235.88	3.52	99.09	0.01	3100.83	0.31
GL-6	2011.51	0.20	2216.79	0.22	820.70	0.08	35624.00	3.56	96.54	0.01	2822.02	0.28
GL-7	3486.98	0.35	2509.97	0.25	3492.65	0.35	32153.80	3.22	175.33	0.02	4622.47	0.46
GL-8	3242.71	0.32	2431.89	0.24	2903.58	0.29	31205.43	3.12	209.54	0.02	4843.27	0.48
GL-9	3578.69	0.36	2481.48	0.25	1076.76	0.11	38587.03	3.86	145.45	0.01	2424.49	0.24
GL-10	3140.74	0.31	2213.01	0.22	1130.24	0.11	37656.15	3.77	136.82	0.01	2113.39	0.21
GL-11	2163.60	0.22	2969.69	0.30	1704.69	0.17	39676.10	3.97	342.81	0.03	1064.47	0.11
GL-12	1964.03	0.20	2847.49	0.28	1758.35	0.18	38615.25	3.86	272.77	0.03	827.14	0.08
GL-13	1258.38	0.13	2128.50	0.21	2946.04	0.29	27479.14	2.75	92.04	0.01	382.50	0.04
GL-14	1059.01	0.11	2148.13	0.21	2997.85	0.30	27238.09	2.72	112.24	0.01	349.87	0.03
GL-15	3256.77	0.33	1902.25	0.19	2384.01	0.24	23904.31	2.39	168.00	0.02	1603.83	0.16
GL-16	2822.50	0.28	1855.30	0.19	2669.17	0.27	23956.49	2.40	160.49	0.02	1537.79	0.15
GL-17	1548.34	0.15	807.83	0.08	940.84	0.09	18463.85	1.85	220.08	0.02	2819.72	0.28
GL-18	4467.81	0.45	2056.78	0.21	919.54	0.09	25939.55	2.59	271.18	0.03	2329.20	0.23
GL-19	2248.12	0.22	1870.38	0.19	460.37	0.05	19707.66	1.97	158.82	0.02	1837.87	0.18
GL-20	4402.56	0.44	2259.61	0.23	1775.08	0.18	25768.45	2.58	249.93	0.02	1523.53	0.15

GL-1 (Cultivated, Pian Perduto); GL-2 (Cultivated, Pian Perduto); GL-3 (wild, Pian Grande); GL-4 (wild, Pian Grande); GL-5 (wild, Santa Maria Maddalena); GL-6 (wild, Santa Maria Maddalena); GL-7 (wild, Pian Perduto); GL-8 (wild, Pian Perduto); GL-9 (wild, Prati di Ragnolo); GL-10 (wild, Prati di Ragnolo); GL-11 (wild, Fargno); GL-12 (wild, Fargno); GL-13 (commercial, Varnelli company); GL-14 (commercial, Varnelli company); GL-15 (commercial, Camerino); GL-16 (commercial, Camerino); GL-17 (commercial, Foggia); GL-18 (commercial, Castelraimondo); GL-19 (commercial, Matelica); GL-20 (commercial, Ascoli Piceno).

percent of recovery was evaluated by calculating the ratio of detected amount versus the added amount; the mean recovery of each compound is shown in Table 1. Calibration curves were constructed by injecting standard mixture solutions at the eight concentrations of 0.5, 1, 5, 10, 25, 50, 75 and 100 μg/ml. The LODs and LOQs were obtained by injecting serial dilutions of the corresponding standard solutions, taking the signal-to-noise (S/N) ratio of 3 and 10 as criteria, respectively. The method revealed good precision with inter and intra-day variations where RSD (%) ranged from 0.24 to 1.43 and 0.23 to 1.23, respectively. The LODs ranged from 0.02 to 0.25 μg/ml, while the LOQs were defined in the range of 0.05 to 0.75 μg/ml. The six analytes demonstrated good linearity ($R^2 \geqslant 0.9997$) in a wide concentration range. The influence of the matrix on the analytes quantification was evaluated, but no significant matrix effect was observed for each molecule.

3.2.2. HPLC/ESI-MS

HPLC/ESI-MS method was also fully validated in terms of precision, accuracy, linearity, limits of detection (LODs) and limits of quantification (LOQs) as described above for the HPLC/DAD method (Table 1). The method showed good precision, demonstrated by the RSD (%) of the inter and intra-day studies, which ranged from 1.41 to 4.80 and 2.97 to 4.61, respectively. The percent of recovery in spiking study (Table 1) was within the accepted limits, indicating good accuracy of the method, and all the analytes demonstrated good linearity ($R^2 \geqslant 0.9992$). The HPLC/ESI-MS method was more sensitive, as LODs and LOQs ranged from 0.01 to 0.025 μg/ml and 0.03 to 0.075 μg/ml, respectively, which are lower than the corresponding values of HPLC/DAD. For these reasons we decided to apply HPLC/ESI-MS method for the subsequent quantitative analysis of the gentian samples. The calibration ranges

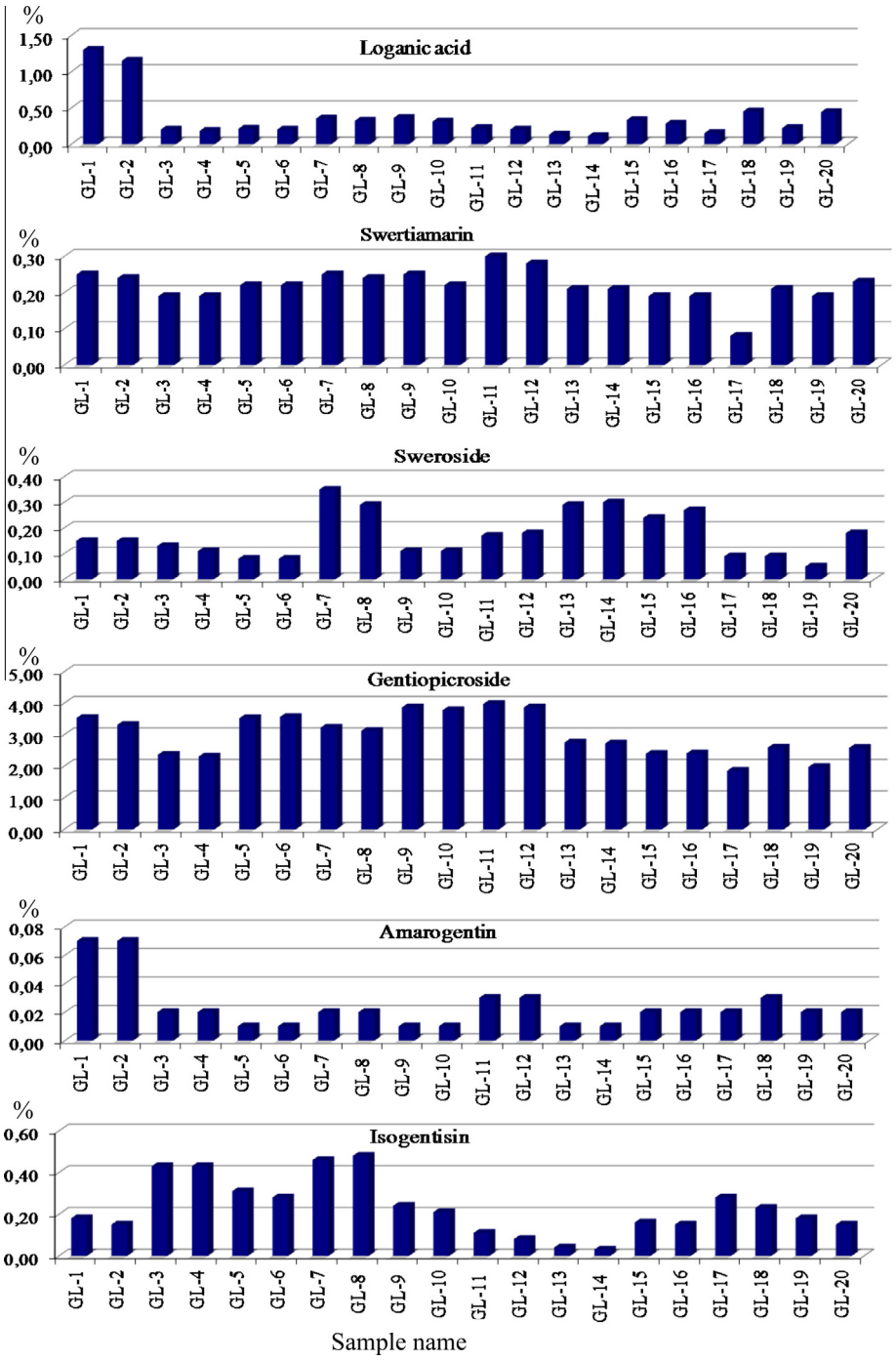

Fig. 3. Schematic diagram representing concentrations of loganic acid, swertiamarin, sweroside, gentiopicroside, amarogentin and isogentisin in different *Gentiana lutea* L. samples.

adequately covered the variations in the amounts of the standards in the samples during analysis.

3.3. Application of the HPLC/ESI-MS method to gentian samples

Since the chromatographic peaks could not be identified unambiguously only by retention time and UV spectra in HPLC, we used HPLC/ESI-MS as a supplement not only for confirmation of peak identification, by comparing the retention time and molecular ion or mass spectra, but also for quantification, by comparing the peak areas of the compounds identified with those of the standards in EIC (Table 2). In this study, the HPLC/ESI-MS method we developed was successfully applied to analyse 20 samples of *G. lutea*. for their quantity of the analysed compounds. The highest content secoiridoid in the Gentian plant is gentiopicroside, with bitterness value of 12,000, while the lowest content secoiridoid, amarogentin, has a bitterness value of 58,000,000. In fact, amarogentin is considered the most bitter substance known to man and is used in soft drinks as an alternative bittering agent to quinine, which has a bitterness value of only 200,000 (Capasso, Gaginella, Grandolini, & Izzo, 2003; European Medicines Agency, 2009; Keil, Härtle, Guillaume, & Psiorz, 2000). Also, secoiridoid glycosides contribute to the gastroprotective effects of Gentian root on gastric lesions, as these glycosides are associated with enhanced mucosal defensive factors via the prostaglandin pathway in the cell membrane (Niiho et al., 2006). Loganic acid (iridoids) and isogentisin

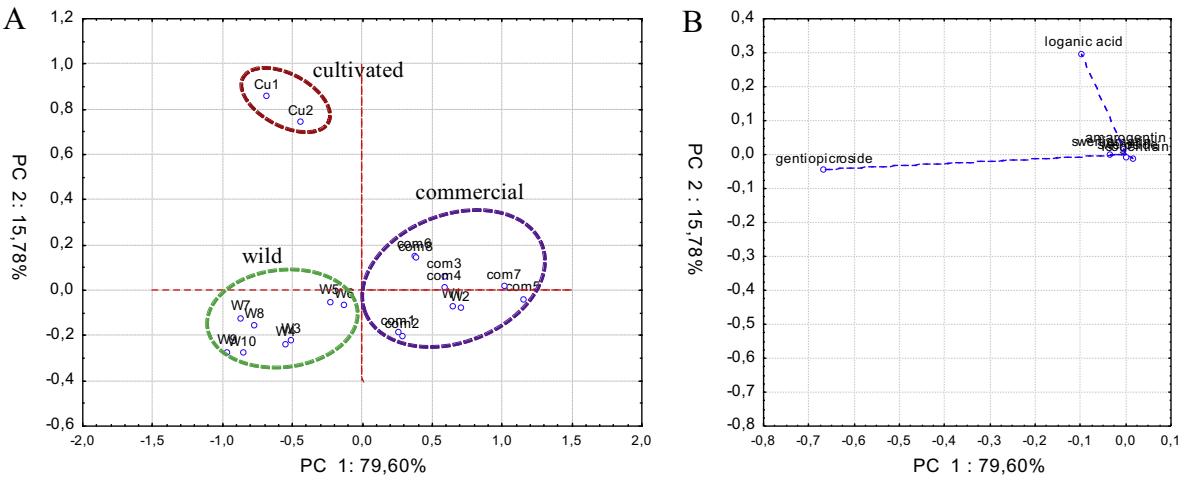

Fig. 4. (A) Score plot (PCA) for main variation of *Gentiana lutea* L. samples. (B) The PCA loading plot for contents of the analysed compounds.

(xanthones) are also biologically active as anti-inflammatory and potent MAO inhibitor, respectively.

The analysed samples showed a content of gentiopicroside, the major bitter principle of the gentian plant, that ranged from 1.85% to 3.97% (Table 2, Fig. 3), with the highest quantity (3.97%) observed in GL-11 (wild) and the lowest (1.85%) in GL-17 (commercial). Gentiopicroside was present at high percentages in samples GL-1, GL-4, GL-5, GL-6, GL-9, GL-10, GL-11, GL-12. The levels of the bitterest natural product identified to date, amarogentin, ranged from 0.01% to 0.07%, with the highest content (0.07%) observed in GL-1 (cultivated) and the lowest (0.01%) in GL-13 (commercial). The contents of swertiamarin and sweroside ranged from 0.08% to 0.30% and 0.05% to 0.35%, respectively. Loganic acid showed a high percentage (1.30%) in cultivated samples (GL-1, GL-2) compared to wild and commercial samples, more than the percentage reported in literature (Aberham et al., 2011, 2007). The levels of isogentisin ranged from 0.03% to 0.48%. According to the results we obtained, the cultivated samples (GL-1, GL-2) were the richest ones in loganic acid and amarogentin and contained high percentages of gentiopicroside, quite close to wild samples. This indicates that the *G. lutea* cultivated in the Monti Sibillini National Park (Italy) for the first time is an excellent source for production of high quality plant material. Fig. 3 represents concentrations of loganic acid, swertiamarin, sweroside, gentiopicroside, amarogentin and isogentisin in different *G. lutea* samples.

Finally, analysis of twenty samples of cultivated, wild and commercial *G. lutea* L. showed that gentiopicroside is the most dominant compound (1.85–3.97%), followed by loganic acid (0.11–1.30%), isogentisin (0.03–0.48%), sweroside (0.05–0.35%), swertiamarin (0.08–0.30%), and finally amarogentin (0.01–0.07%). These results are in a good agreement with the reported data (Aberham et al., 2011, 2007; Yang et al., 2009). The differences in the content of constituents between cultivated and wild samples could be due to the different altitudes at which they were collected, while in the case of commercial samples the provenience and the methods of drying and handling could be responsible for the differences of concentration.

3.4. Principal component analysis (PCA)

The PCA made it possible to differentiate the 20 samples of *G. lutea* on the basis of their content of secondary metabolites. The 2D graphical representation of principal-component analysis is shown in Fig. 4, and represents 95.4% of the total variance in the data set. The variability of data was generated mostly by the content of gentiopicroside (values of eigenvectors: −0.66; −0.04) in the first PC and by loganic acid (values of eigenvectors: −0.09; 0.29) in the second PC. Samples on the lower left hand side of the PCA score plot (Fig. 4A) came from wild populations (apart from samples W1 and W2) which are characterised by high levels of gentiopicroside (Fig. 4B). Samples on the top left hand side (Fig. 4A) came from experimental cultivations, which were characterised by high levels of loganic acid and, to a lesser extent, by gentiopicroside (Fig. 4B). Finally, samples on the middle right hand side of the PCA score plot (Fig. 4A) were purchased commercially, and correlated to the other secondary metabolites such as amarogentin, swertiamarin, sweroside and isogentisin. In conclusion, the variability found in the data matrix seems to be correlated to the different origin of the samples. The high content of gentiopicroside appears to be characteristic of wild-type roots; high levels of loganic acid and gentiopicroside characterised cultivated samples. Finally the commercial samples seem to be characterised by lower levels of loganic acid and gentiopicroside.

4. Conclusions

The present work is the first report of an accurate, fast and reliable analytical method for the simultaneous quantitation of iridoids (loganic acid), secoiridoids (gentiopicroside, sweroside, swertiamarin, amarogentin) and xanthones (isogentisin) in different populations of cultivated, wild and commercial *G. lutea* by HPLC/ESI-MS. This method is fully validated and offers good linearity, accuracy, repeatability and precision, and thus the HPLC/ESI-MS under our experimental conditions represents a valuable method for the qualitative and quantitative assay of the most important components of *G. lutea* This new method should prove useful and reliable for quality control of the *G. lutea* plant and its related products. The HPLC results and follow-up PCA analysis confirmed that the *G. lutea* we have cultivated for the first time in the Monti Sibillini National Park (Italy) could be an excellent source for production of high quality plant material due to its high contents of the analysed compounds, compared to wild and commercial samples.

Conflict of interest

None of the authors has any conflict of interests that could affect the performance of the work or the interpretation of the data.

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